Chapter 21
Poxviral TNFRs: Properties and Role in Viral Pathogenesis

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Poxviruses

Poxviruses are a family of large irregularly shaped dsDNA viruses replicating exclusively in the cytoplasm of the infected cell that include a number of human and animal pathogens [13]. Most notorious amongst these is the fortunately eradicated variola virus (VARV), a highly specific and contagious human virus that caused smallpox, a disease with mortality rates of up to 30% that has caused major pandemics recorded as early as 1350 BC. Other poxviruses causing disease in humans include monkeypoxvirus (MPXV), an emerging zoonotic disease detected in African countries as well as in the USA with mortality rates of up to 10% [9], molluscum contagiosum virus, which causes benign tumorations mainly in children, and yaba-like disease virus that causes a mild, self-limiting illness. The prototypical member of this virus family is vaccinia virus (VACV) which was used as a live attenuated vaccine for the eradication of smallpox. Lately, recurrent human infections from circulating VACV-derived viruses adapted to animal hosts have been detected in India and Brazil, suggesting the potential for adaptation and re-emergence of these viruses. Additionally, poxviruses are currently under study for the development of vaccines and use in oncolytic therapies. Therefore, comprehension of the mechanisms of pathogenesis and a better understanding of the host immune modulation strategies used by poxviruses are essential.

Poxviral TNF-Binding Proteins

Concurrently with the first identification and molecular cloning of the human TNFRs in 1990 [11, 22, 27], Smith and colleagues identified a putatively secreted viral protein termed T2 in Shope fibroma virus, a poxvirus infecting rabbits, with

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similarity to the TNFR family. Indeed, they proposed that this could act as a soluble TNF or related cytokine receptor and that such a protein "would no doubt confer a selective advantage to the pathogen" [27]. Since then, most poxviruses have been shown to encode at least one predicted active secreted TNF-binding protein, which can be divided into two groups based on their primary sequences. A class of these TNF inhibitors termed viral TNFRs (vTNFRs) are homologues to the extracellular domain of host TNFRs, which are characterized by the presence of a variable number of copies of the cysteine-rich domain (CRD) that defines the TNFRSF. These include the mentioned M-T2 protein (and its orthologues present in other viruses of the Leporipoxvirus genus), four different proteins named cytokine response modifier B (CrmB), CrmC, CrmD, and CrmE, which are differentially expressed by members of the poxvirus family, as well as a viral homologue of the TNFRSF member CD30. A second class of secreted viral TNF-binding proteins have no amino acid sequence similarity to known cellular proteins and include the 2L protein of the yatapoxviruses and their swinepox and deer poxvirus orthologues.

With the exception of the viral CD30 homologue, which was shown to bind exclusively to cellular TNFSF8 and whose role in pathogenesis is not clearly defined [3, 15, 21], all other proteins mentioned were found to bind specifically and with high affinity to TNF and in some cases LT alpha of at least the species infected by the corresponding virus where they were derived from (see Table 21.1 for a succinct description of the main properties of these proteins).

The myxoma virus M-T2 protein is most similar to the cellular TNFR2. It contains four N-terminal CRDs, followed by a C-terminal domain that shows no similarity to cellular genes. This secreted protein was found to bind to rabbit TNF with high affinity through its four N-terminal CRDs and inhibit its activity in cell culture [23]. Additionally, M-T2 was described as a virulence factor during the development of myxomatosis in infected European rabbits. More recently, the intracellular pool of M-T2 was shown to have an important role, as it can inhibit TNF-mediated signaling through PLAD domain-mediated sequestration of cellular TNFR1, acting effectively as a dominant negative mutant that prevents virus-induced apoptosis independently of its TNF-binding capability [23–26]. Probably both activities act in concert in vivo to provide its, however, relatively minor role in pathogenesis.

The orthopoxviral CrmC and CrmE proteins are composed of only three CRDs each and, although most similar to the cellular TNFR2, have been found to bind to TNF but not LT. The possibility that VACV CrmE may act at the cell surface, by interacting with the cell membrane after secretion, as well as in solution has been raised [18]. The solution of the crystal structure of the VACV CrmE protein showed that it adopts a canonical TNFR fold. Although the structure of CrmE complexed with TNF was not reported, comparison of CrmE with the TNFR structure suggests that it binds to TNF using the 50s and 90s loops present on CRDs numbers 2 and 3, respectively. At the moment, this is the only member of the vTNFR family whose structure has been solved and it may be used as a model to understand the ways in which these proteins are able to bind to and inhibit their ligands. Thus, it has been proposed that the 90s loop is involved in fine-tuning ligand specificity and affinity,

Table 21. 1 Main properties of the poxviral TNF-binding proteins

Protein	Virus (virus family)	Known ligands	Role in vivo	Reference(s)
2L	Tanapox virus (yatapoxvirus)	TNF β2microglobulin	Unknown	[5, 16, 17, 24, 26, 28, 30]
T2	Shope fibroma virus, myxoma virus (leporipox virus)	TNF, LTα TNFR1	Virulence factor	
CrmB	VARV, MPXV, CPXV, CMPV, HSPV (orthopoxvirus)	TNF, LTα, chemokines	Anti-inflammatory, virulence factor	[1, 2, 8, 10, 14, 29]
CrmC	CPXV, VACV some strains only (orthopoxvirus)	TNF	Virulence factor	
CrmD	ECTV, CPXV (orthopoxvirus)	TNF, LTα, chemokines	Unknown	[2, 12]
CrmE	CPXV, VACV some strains only (orthopoxvirus)	TNF	Virulence factor	[18, 21]
vCD30	ECTV, CPXV (orthopoxvirus)	TNFSF8	Modulates Th1 responses in vivo, not major virulence factor during infection	[3, 15, 21]

whereas the 50s loop may determine the binding affinity of these viral receptors [9]. Finally, the CrmB and CrmD proteins share a domain structure similar to that of the M-T2 protein, with four N-terminal CRDs followed by a C-terminal domain unrelated to TNFRs. Both are secreted proteins and bind with high affinity to TNF and LT, preventing their biological activity in cell culture assays. Interestingly, these proteins were found to be able to bind to a reduced set of chemokines (chemotaxis-inducing cytokines) through their C-terminal domain, which has been named the SECRET domain (for Smallpox virus-Encoded Chemokine REcepTor) and inhibit their biological activity in cell culture [2]. The lack of amino acid sequence similarity of the SECRET domain with host chemokine receptors and other virus-encoded chemokine-binding proteins suggests that this domain represents a new protein structure able to bind chemokines with high affinity. The TNF and chemokine ligands were shown to be bound simultaneously through structurally independent domains of the VARV CrmB and ECTV CrmD proteins, which were proposed to act as immunomodulatory proteins with dual activities in vivo. Whether this property may be shared by the M-T2 protein remains to be addressed.

vTNFRs in Pathogenesis

Somewhat surprisingly given the importance of orthopoxviral infections in humans, the evidence on the contribution of orthopoxviral vTNFRs to virulence in vivo is scarce so far.

The CPXV CrmB protein was proposed to have anti-inflammatory activity in vivo using a chicken embryo chorioallantoic membrane infection model and the CrmB deletion mutant CPXV showed an approximately 50-fold reduced LD_{50} in intracranially infected mice [14]. However, the CPXV strain used still contained other active vTNFRs, impairing a clear attribution of TNF or chemokine inhibitory activities to the observed phenomena. Additionally, the model of intracranial infection used can hardly mimic the natural route of infection. More recently, expression of CPXV CrmB, VACV CrmC or CrmE proteins from a recombinant attenuated VACV was shown to increase the virulence using an intranasal mouse model of infection, but not an intradermal infection model. Deletion of the VACV CrmE, but not CrmC, did similarly attenuate viral infection in mice using the intranasal model [18]. This evidence is limited to models that required high virus doses to cause disease and the effects observed are relatively minor. Lastly, no description of the possible role of the CrmD protein in vivo has been published yet. Therefore, we believe that better in vivo models may be necessary for the study of the contribution of vTNFRs to viral pathogenesis and immune regulation.

Ectromelia Virus

Ectromelia virus (ECTV) is an orthopoxvirus that has host specificity for the mouse and is genetically very similar to VACV, VARV, and MPXV [7]. Susceptible strains of mice infected with ECTV develop mousepox that, like smallpox, is a severe disease with high mortality and infectivity rates, and constitutes an excellent model to study smallpox. The fact that infection with ECTV in susceptible strains of mice rapidly becomes systemic when inoculated via the subcutaneous or intranasal route has been exploited not only as a tool to investigate the pathogenesis and immunology of poxvirus infections, but also as a model of generalized virus infections, genetic resistance to disease, and viral immunology [7].

Previous data suggested an important role for TNF-induced signaling in mousepox pathogenesis, as transgenic-resistant mice lacking functional TNFR1 and TNFR2 became susceptible to ECTV, with highly increased viral titers in the main target organs, liver and spleen, of the infected mice [20]. Moreover, treatment of susceptible BALB/c mice with murine TNF was able to hinder ECTV replication and mortality to some extent [4]. Importantly, ECTV encodes only one active vTNFR, the CrmD protein, which is expressed as a secreted protein at late times postinfection. VARV, the causative agent of human smallpox, does similarly encode only one secreted vTNFR predicted to be active, the CrmB protein. As mentioned before, both proteins have been characterized and shown to have similar binding properties

in vitro. For these reasons, ECTV constitutes an excellent model for the study of the role of vTNFRs in the pathogenesis of orthopoxviral infections.

To address this issue, we have constructed a recombinant ECTV lacking the gene encoding CrmD and studied its virulence in vivo. Experiments performed in cell culture confirmed that this deletion mutant did not express any detectable soluble TNF-binding activity and that deletion of CrmD did not alter the replication capacity of the virus. When infected into susceptible BALB/c mice, however, the CrmD deletion mutant was found to be severely attenuated, with an LD_{50} reduced by at least six orders of magnitude. Moreover, absence of CrmD was accompanied by a strong and quick inflammatory response at the initial infection site and reduced viral spread to the main target organs, spleen and liver. This identifies CrmD as one of the main determinants of mousepox virulence in vivo, suggesting that the related CrmB protein found in VARV might be of similar importance during the development of human smallpox. Interestingly, the TNF-regulated response in macaques experimentally infected with VARV was impaired as compared to infections with other viruses [19], suggesting an active viral TNF-blocking mechanism which may be mediated by the CrmB protein, amongst others.

Given that CrmD is able to block both TNF and chemokines simultaneously, we propose a working model (see Fig. 21.1) in which blockade of TNF hinders the establishment of a proinflammatory state locally, while chemokine blockade further impairs the ensuing recruitment of leukocytes to the initial infection site. This may allow enhanced replication and spread of the virus through the infected host by preventing the establishment of an adequate antiviral immune response in vivo.

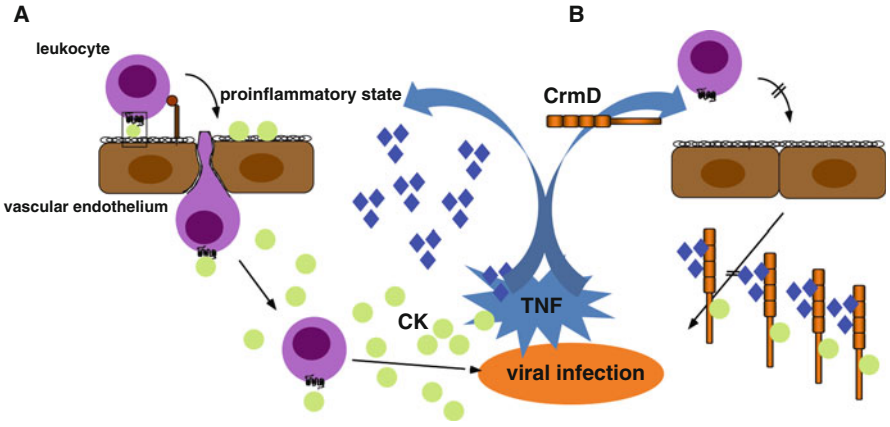

Fig. 21.1 Proposed working model for the CrmD protein. (**a**) In response to viral infection, TNF and other proinflammatory cytokines are produced. This proinflammatory state promotes the expression of chemokines (CK) that will recruit leukocytes to the site of infection to eliminate or control the incoming virus. (**b**) In the presence of the secreted TNF and CK inhibitory protein CrmD expressed by ECTV infected cells, this reaction is blocked in two steps by first impairing the establishment of a proinflammatory state and later directly preventing leukocyte migration

To further understand the molecular mechanisms of CrmD activity during infection, we are currently studying in greater detail the structural determinants of CrmD interactions with its TNF/LT and chemokine ligands. Our aim is to generate recombinant ECTV expressing mutant versions of the protein binding to only one of its known ligands at a time. This will allow us to determine the individual contribution of these cytokines in the establishment of the antiviral response. This approach will also help us to understand the structural basis of the CrmD–ligand binding that could be used as a model to study molecularly the TNFSF–TNFRSF interaction, since surprisingly, despite the importance of these cytokines in the immune response, there are few data about the structural features of the ligand–receptor complexes.

Conclusions

In this study, we demonstrate that CrmD is an essential virulence factor, as deletion of CrmD from ECTV resulted in an avirulent virus that induced an acute early inflammatory response at the initial site of infection but did not establish a fatal disease. This is one of the most dramatic attenuation phenotypes described in poxviruses after inactivation of a viral immune modulatory activity and demonstrates a critical role of TNF and a reduced set of chemokines in antiviral defence. Moreover, this unique experimental model of virus infection will allow us to dissect in vivo the relative contribution of TNF and chemokine activities to the establishment of an adequate immune response.

Anti-TNF therapy, based on either monoclonal antibodies or soluble TNFRs, is available in the clinic as an efficient way of blocking adverse inflammatory responses. A better knowledge of the strategies used by viruses to modulate TNF activity during infection may uncover new anti-TNF strategies that could be applied to the clinic. The use of soluble versions of TNFRs by viruses confirms an adequate strategy used in the clinic. A better understanding of the function of the different vTNFRs in vivo may help us to improve even further the potency of soluble TNFRs in blocking adverse inflammatory responses in a number of human disease conditions.

The investigations described here on viral strategies to modulate the activity of TNF illustrate how a better understanding of the interaction of viruses with the host immune system may provide information relevant to viral pathogenesis and suggest new strategies of immune modulation for the treatment of human disease.

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